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Roles of *N*-Acetylglutaminylglutamine Amide and Glycine Betaine in Adaptation of *Pseudomonas aeruginosa* to Osmotic Stress

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The mechanism of osmotic stress adaptation in *Pseudomonas aeruginosa* PAO1 was investigated. By using natural abundance ^{13}C nuclear magnetic resonance spectroscopy, osmotically stressed cultures were found to accumulate glutamate, trehalose, and *N*-acetylglutaminylglutamine amide, an unusual dipeptide previously reported only in osmotically stressed *Rhizobium meliloti* and *Pseudomonas fluorescens*. The intracellular levels of these osmolytes were dependent on the chemical composition and the osmolality of the growth medium. It was also demonstrated that glycine betaine, a powerful osmotic stress protectant, participates in osmoregulation in this organism. When glycine betaine or its precursors, phosphorylcholine or choline, were added to the growth medium, growth rates of cultures in 0.7 M NaCl were increased more than threefold. Furthermore, enhancement of growth could be observed with as little as 10 μM glycine betaine or precursor added to the medium. Finally, the mechanism of osmotic stress adaptation of two clinical isolates of *P. aeruginosa* was found to be nearly identical to that of the laboratory strain PAO1 in all aspects studied.

The ability of bacteria to adapt to hyperosmotic conditions allows them to survive in a variety of hostile environments such as relatively dry surfaces or saline fluids. Adaptation to high osmolality (osmoregulation) is generally accomplished by the intracellular accumulation of organic compounds which are compatible with macromolecular function (23). These compounds, called osmolytes, fall into a few classes: polyols (sugars, glycerol), amino acids (proline, glutamate), amino acid derivatives (glycine betaine and gamma-aminobutyric acid), peptides, methylamines, and urea (5, 18, 23). Osmolytes such as glutamate or trehalose can be synthesized by microbes, while others are accumulated by transport from the medium (5). A prominent example of the latter is the osmoregulated transport and intracellular accumulation of glycine betaine, which is reported to protect a number of bacterial species against osmotic stress (4).

The focus of this investigation is the mechanism of osmotic stress adaptation in the opportunistic pathogen *Pseudomonas aeruginosa*. Although *P. aeruginosa* infection can occur in patients that are immunocompromised because of a variety of conditions (6), it is commonly seen in patients suffering from cystic fibrosis (CF). Indeed, chronic lung infection by *P. aeruginosa* is one of the most critical problems facing CF patients today (9). This type of infection is also a remarkable example of adaptation to osmotic stress. The CF lung environment is especially osmotically stressful, with viscous, dehydrated mucus, cellular debris, and electrolytes all competing for a limited supply of moisture (3, 17). Yet, knowledge of the mechanism by which *P. aeruginosa* adapts to high osmolality is largely lacking (4). Furthermore, recent reports on *P. aeruginosa* are beginning to link osmotic stress to pathogenicity itself. For example, high osmolality is one of the signals controlling the synthesis of the virulence factor alginate (1, 7). Also, production of the virulence factor phospholipase C is induced by the osmotic stress protectant glycine betaine and its derivatives (19).

In this article, we identify the major osmolytes synthesized and accumulated by *P. aeruginosa* PAO1 grown at high osmotic strength. We also show that the bacterial response to osmotic stress is modulated by glycine betaine and its derivatives.

MATERIALS AND METHODS

Materials, bacterial strains, and media. *P. aeruginosa* PAO1 was kindly provided by John Ingraham, University of California, Davis. Clinical isolates of *P. aeruginosa* originated from sputum cultures of CF patients and were provided by Ruth McDonald, Cystic Fibrosis Center, University of California, Davis. Strains were maintained on solid media containing Pseudomonas Agar F (Difco Laboratories). Inocula were grown in YTA medium (1) containing 5 g of yeast extract, 10 g of tryptone, and 2 g of glucose, each per liter. The minimal medium used was VB medium (22), which contained 5 g of glucose per liter and 3.5 g of $Na(NH_4)HPO_4 \cdot 4H_2O$ per liter as the C and N sources, respectively. In certain experiments, either 5 g of glycerol per liter or 16 mM glutamate was substituted for the glucose or the ammonium ion, respectively, contained in the VB medium. Glycine betaine and its derivatives were sterilized by filtration. The osmolalities of the media were measured by freezing-point depression with a wide-range osmometer (model 3W; Advanced Instruments, Inc.). [^{14}C]inulin (3.15 mCi/g) and [^{14}C]sorbitol (315 mCi/mmol) were from ICN Biomedicals, Inc. ^3H_2O (1 mCi/g) and Aquasol were from NEN Research Products. All other chemicals used were reagent grade or the best grade available.

Growth measurements. Cultures were grown in YTA medium, subcultured into VB medium, grown to late log or

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early stationary phase, and used to inoculate (3%) the VB medium used for measurements of growth. Growth rates were determined by turbidity by using a Klett-Summerson colorimeter with a no. 54 filter. Cultures were grown with shaking at 37°C, and all experiments were repeated at least once, with about 5 to 10% error.

Preparation of cell extracts for NMR spectroscopy. Cultures were subcultured and grown as described above. One to 2 liters of culture was harvested during log phase (approximately 200 to 250 Klett units) and extracted with perchloric acid as described previously (20). ^{13}C nuclear magnetic resonance (NMR) spectra were obtained at 75.57 MHz with a General Electric NMR Omega-300 spectrometer. A Waltz 16 decoupler modulation was used to obtain broad-band proton decoupling. Spectra were acquired by using a 70° flip angle, a 3-s pulse repetition rate, a 20-KHz spectral window, and 8,192 complex time domain datum points. Exponential multiplication producing a 5-Hz line broadening was used before Fourier transformation of each spectrum. Each sample required 1,024 acquisitions, and other NMR parameters are described elsewhere (20). The concentrations of osmolytes were quantitated by comparison with an internal standard (100 μmol of alanine). In some samples, the quantitation of glutamate was verified by amino acid analysis. Chemical shifts are reported relative to tetramethylsilane, assuming that dioxane in 15% D_2O has a chemical shift of 67.3 ppm. All samples were run at least in duplicate, with an error in quantitation of about 10 to 20%. The intracellular osmolyte content is reported per milligram of cellular protein, which was determined by measuring the turbidity with a Klett-Summerson colorimeter. Under these conditions, 100 Klett units is equivalent to 87 μg of protein per ml of culture by the method of Lowry et al. (15).

Measurement of cellular volume. The cellular volume of osmotically stressed and unstressed cells was determined by the method of Stock et al. (21) as modified by Cayley et al. (2), except that [^{14}C]sorbitol was used in place of [^{14}C]sucrose. In this procedure, the volumes in a cellular pellet accessible to ^3H_2O, [^{14}C]inulin (excluded from the cell), and [^{14}C]sorbitol (excluded from the cytoplasm but not periplasm) are compared to obtain water-accessible cellular and cytoplasmic volumes. Radioactive samples were added to 15 ml of Aquasol and analyzed by liquid scintillation counting by using a Beckman LS 7500 liquid scintillation counter.

RESULTS

Osmolyte composition of *P. aeruginosa* PAO1. Natural abundance ^{13}C NMR spectroscopy was employed to identify all osmolytes that accumulate in salt-stressed *P. aeruginosa*. This technique is particularly useful because any carbon-containing osmolyte accumulating to significant levels can be detected regardless of its structure or chemical reactivity. Three principal osmolytes were observed in ^{13}C NMR spectra of perchloric acid extracts of osmotically stressed *P. aeruginosa* PAO1: *N*-acetylglutaminylglutamine amide (NAGGN) (179.7, 177.6, 176.3, 175.5, 55.1, 54.7, 32.9, 28.4, and 23.5 ppm), glutamate (182.0, 176.5, 56.5, 35.2, and 28.8 ppm), and trehalose (94.9, 74.3, 73.8, 72.8, 71.5, and 62.3 ppm). A low level of glucose was also observed, but this sugar also occurred at similar levels in spectra of extracts from unstressed cultures and thus appeared not to be osmoregulated. It may have originated, in part, from glucose in the growth medium that was trapped in the intercellular spaces of the harvested culture.

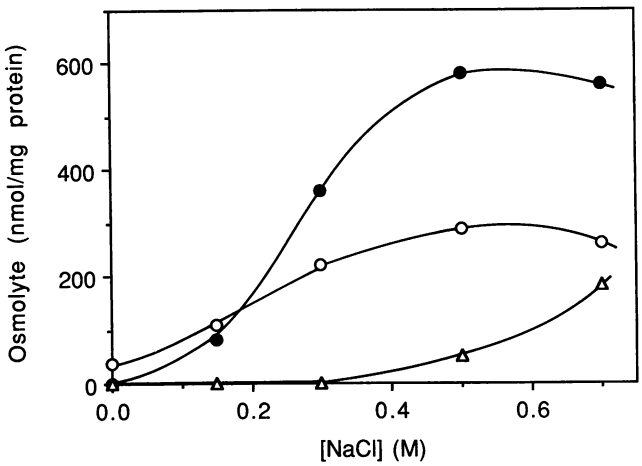

FIG. 1. Intracellular osmolyte accumulation as a function of NaCl in the growth medium. *P. aeruginosa* PAO1 was grown in VB medium containing NaCl as indicated, and osmolytes were quantitated by ^{13}C NMR spectroscopy as described in Materials and Methods. Osmolytes observed were NAGGN (●), glutamate (○), and trehalose (△).

Osmotic regulation of NAGGN, glutamate, and trehalose. The control of osmolyte accumulation by medium osmolality was examined further by determining the intracellular levels of NAGGN, glutamate, and trehalose as a function of NaCl in the medium. Figure 1 shows that the levels of all three osmolytes increased as the concentration of NaCl was increased. At low NaCl concentrations, the increases in glutamate and NAGGN were about the same; however, above 0.15 M NaCl, the increase in the intracellular content of NAGGN was up to twofold greater than that of glutamate, so that NAGGN became the dominant osmolyte. Trehalose accumulation was significant only in highly stressed cells. More than 0.3 M NaCl must be added to the medium for trehalose to be observed, and its intracellular level became pronounced only in cells stressed above 0.5 M NaCl. In the absence of NaCl added to the medium, NAGGN and trehalose were undetectable, and only a low level of glutamate was observed, indicating that all three compounds are osmoregulated.

Cultures were also grown in a variety of solutes, and the levels of osmolytes were measured. In this way it could be determined if the accumulation of osmolytes observed in *P. aeruginosa* was due to the osmotic strength of the medium or to specific Na^+ or Cl^- effects. The pattern of osmolyte accumulation from cultures stressed with the inorganic solutes KCl, K_2HPO_4, or K_2SO_4 or with sucrose was similar to that obtained with NaCl (Table 1). To rule out the possibility that osmolyte accumulation was dependent on the glucose or the ammonium ion in the medium, cultures were grown either with glycerol or glutamate as the C or N source, respectively (Table 1). Results with glycerol were similar to those with glucose, indicating that osmolyte accumulation is independent of the glucose in the medium. However, glutamate added to the medium resulted in a threefold increase in trehalose content and a twofold decrease in NAGGN content. Hence, in all media tested, NAGGN, glutamate, and trehalose were observed. But when glutamate was added to the medium as the N source, it caused a shift from NAGGN to trehalose as the principal osmolyte, a point that is discussed below.

TABLE 1. Osmolytes accumulated by osmotically stressed *P. aeruginosa* PAO1[a]

Addition to medium	Osmolality (osmol/kg of H_2O)	Accumulated osmolyte (nmol/mg of protein)		
		NAGGN	Glutamate	Trehalose
None	0.21	<10	38	<10
0.7 M NaCl	1.54	560	260	180
0.7 M KCl	1.57	210	140	71
0.45 M K_2HPO_4	1.53	330	270	190
0.65 M K_2SO_4	1.76	240	130	130
0.9 M Sucrose	1.46	680	280	280
0.7 M NaCl (glycerol[b])	1.57	450	320	250
0.7 M NaCl (glutamate[c])	1.53	270	570	610

[a] Cultures were grown in VB medium with the additions shown. The levels of osmolytes were determined by ^{13}C NMR spectroscopy by using perchloric acid extracts of cultures grown to log phase.
[b] Glycerol was substituted for glucose as the C source in VB medium.
[c] Glutamate was substituted for ammonium ion as the N source in VB medium.

The intracellular concentrations of osmolytes can be calculated from the values reported in Table 1, if the cellular volumes are known. By using the radiolabeling technique of Stock et al. (21) (see also reference 2), the water-accessible volumes of unstressed cells and cells stressed with 0.7 M NaCl were determined. The volumes of unstressed cells and NaCl-stressed cells were found to be 4.92 and 2.97 μl/mg of protein, respectively. Therefore, in unstressed cultures, the intracellular concentration of the only observable osmolyte, glutamate, was 7.7 mM. At 0.7 M NaCl, the intracellular concentrations of NAGGN, glutamate, and trehalose were 188, 87, and 61 mM, respectively.

Role of glycine betaine in osmoregulation of *P. aeruginosa*. Limited data which indicate that the growth rate of osmotically stressed *P. aeruginosa* increases if glycine betaine is included in the medium have been published (4). Therefore, it was of interest to investigate more fully the role that this osmotic stress protectant plays in osmoregulation. The ability of glycine betaine to enhance the growth of stressed *P. aeruginosa* was quantitated in cultures grown with from 0 to 1.0 M NaCl (Table 2). While glycine betaine stimulated growth at high osmotic strength, it had no effect when NaCl was omitted from the medium. At 0.7 M NaCl, the growth rate increased about threefold with 1 mM glycine betaine added to the medium. Furthermore, glycine betaine permitted growth of cultures in media containing as much as 1.0 M NaCl. Without glycine betaine, cultures could not grow in media with more than 0.75 M NaCl.

TABLE 2. Effect of exogenously added NaCl and glycine betaine on the growth rate of *P. aeruginosa* PAO1[a]

Concn of NaCl added to medium (M)	Osmolality (osmol/kg of H_2O)	Doubling time (h/generation) with:	
		No addition	Glycine betaine added
0	0.21	1.1	1.1
0.3	0.83	1.6	1.3
0.5	1.12	2.7	1.7
0.7	1.54	8.9	2.6
1.0	2.10	—[b]	13.1

[a] Cultures were grown in VB medium plus NaCl and 1 mM glycine betaine where indicated.
[b] —, no growth observed in 5 days.

TABLE 3. Effect of exogenously added glycine betaine or its derivatives on the growth rate of *P. aeruginosa* PAO1

Compound added[a]	Doubling time (h/generation) in VB medium with:	
	No addition	0.7 M NaCl added
None	0.9	7.9
Glycine betaine	0.9	2.4
Dimethylglycine	1.0	6.0
Monomethylglycine	1.0	7.5
Glycine	1.0	7.0
Choline	1.0	2.8
Phosphorylcholine	0.8	2.2

[a] Glycine betaine and derivatives were added at 0.2 mM.

To characterize the mode of action of glycine betaine on stressed cells, two experiments were carried out. First, the minimum concentration of glycine betaine required for protection of cultures grown in 0.7 M NaCl was determined. Growth rate experiments were carried out with glycine betaine ranging from 0 to 5 mM (data not shown). Interestingly, the protective effect of glycine betaine on growth was observed with as little as 10 μM glycine betaine, and 90% of the maximum effect was achieved with 200 μM glycine betaine. Additional betaine, from 0.5 to 5 mM, did not improve the growth rate. Thus, it seems that *P. aeruginosa* is highly responsive to low levels of glycine betaine in the environment, which will be discussed below.

The second experiment was to determine the specificity of the glycine betaine effect. In this experiment, the demethylated derivatives of glycine betaine, dimethylglycine, monomethylglycine, and glycine were tested for their ability to confer osmotic tolerance. Also, because it is known that *P. aeruginosa* can oxidize choline to glycine betaine (12), the precursors of glycine betaine, choline and phosphorylcholine, were tested. *P. aeruginosa* was grown with or without 0.7 M NaCl and with 0.2 mM glycine betaine or its derivatives added to the growth medium (Table 3). None of the additives significantly altered the growth rate of unstressed cultures. However, when added to the medium at 200 μM (Table 3) or 10 μM (not shown), choline and phosphorylcholine were equal to glycine betaine in the ability to enhance growth of osmotically stressed cultures. Little, if any, enhancement of growth was observed with the demethylated derivatives. The 30% increase in growth rate conferred by dimethylglycine was not due to the intracellular accumulation of dimethylglycine at observable levels (data not shown).

That the ability of glycine betaine to protect the cell against osmotic stress is specific for glycine betaine (and its precursors) and is observable at micromolar concentrations indicates that glycine betaine likely acts as an osmoprotectant and not merely as a nutrient in stressed cultures.

Effect of glycine betaine on the osmolyte composition of the cell. Another line of evidence that would indicate that glycine betaine acts as an osmoprotectant is the observation of its accumulation in the cell. Cultures were grown in VB medium with or without 0.7 M NaCl and with 1 mM glycine betaine. Harvested cells were extracted with perchloric acid, and the osmolytes were identified and quantitated by natural abundance ^{13}C NMR spectroscopy (Table 4). Two results are noteworthy. First, high levels of glycine betaine were observed in stressed cultures, but not in control cultures, consistent with other evidence that betaine acts as an

TABLE 4. Effect of glycine betaine added to the medium on the osmolyte composition of osmotically stressed *P. aeruginosa* PAO1[a]

Addition to the medium	Accumulated osmolyte (nmol/mg of protein)			
	NAGGN	Glutamate	Trehalose	Glycine betaine
None	<10	38	<10	<10
1 mM Glycine betaine	<10	36	<10	50
0.7 M NaCl	560	260	180	<10
0.7 M NaCl and 1 mM glycine betaine	160	300	<15	860

[a] Cultures were grown in VB medium plus additions as shown. The concentrations of osmolytes in the perchloric acid extracts of cultures were determined by ^{13}C NMR spectroscopy.

osmoprotectant. Second, the accumulation of glycine betaine was compensated for by a decrease of all endogenously synthesized osmolytes, which demonstrates that glycine betaine accumulation modulates the intracellular levels of the three endogenous osmolytes. These results and those from growth measurements described above establish the role of glycine betaine as an osmoprotectant in *P. aeruginosa* PAO1.

Osmoregulation of clinical isolates of *P. aeruginosa*. The focus of this research was extended to pathogenic strains to assess the generality of the results described above for the laboratory strain PAO1. In these experiments, four clinical isolates from patients of the Cystic Fibrosis Center were initially examined. Two isolates were not prototrophic in VB medium and were not investigated further. The remaining two isolates (JS and MC) were characterized by NMR spectroscopic analysis and growth rate experiments. Cultures were grown with or without NaCl, and the osmolytes accumulated were identified by NMR spectroscopy as described above for PAO1. At 0.5 M NaCl, the osmolyte compositions of strains JS and MC were similar to that of PAO1 (Fig. 2). The only appreciable difference in osmolyte content was the two- to fourfold increase in trehalose level in the pathogens compared with that in PAO1.

The possibility that glycine betaine could confer enhanced osmotic tolerance to the clinical isolates was also investigated. Cultures were grown in VB medium with 0 to 0.7 M NaCl and 1 mM glycine betaine (Table 5). These strains were not as salt tolerant as PAO1, and, in fact, strain MC could not be reproducibly grown at 0.7 M NaCl. However, the addition of glycine betaine enhanced the osmotic tolerance of *P. aeruginosa* JS and MC. At 0.7 M NaCl, the addition of 1 mM glycine betaine increased growth rates 2.5-fold or more.

The results demonstrate that these clinical isolates and PAO1 responded similarly to high osmolality. They accumulated NAGGN, glutamate, and trehalose when osmotically stressed and utilized glycine betaine as an exogenous osmoprotectant if supplied in the growth medium.

DISCUSSION

In this article, we have shown that the major solutes accumulated in osmotically stressed cultures of *P. aeruginosa* are the dipeptide NAGGN, glutamate, and trehalose. The dominant osmolyte NAGGN was previously reported in *Rhizobium meliloti* and *Pseudomonas flourescens* (20), but in those species glutamate is the most abundant osmolyte. NAGGN was not found in the enterics *Escherichia coli* and

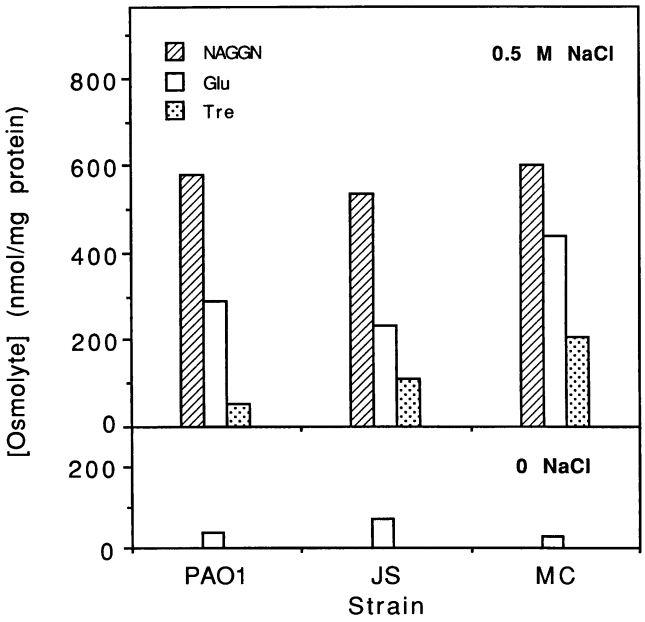

FIG. 2. Identification of osmolytes accumulated by clinical isolates. *P. aeruginosa* PAO1 and clinical isolates (designated JS and MC) were grown in VB medium either with or without 0.5 M NaCl, and osmolytes were identified and quantitated by ^{13}C NMR spectroscopy. In the absence of added NaCl, NAGGN and trehalose were not detected. Glu, glutamate; Tre, trehalose.

Klebsiella pneumoniae (20) or the asymbiotic diazotrophs *Azotobacter chroococcum* and *Azospirillum brasilense* (16). In addition, of the four species examined in the genus *Rhizobium*, only *R. meliloti* (20) was found to contain NAGGN, while both *Pseudomonas* species tested thus far contain this dipeptide. It will be interesting to see how widespread this unusual osmolyte is among eubacteria.

Two lines of evidence suggest that the intracellular accumulation of NAGGN, glutamate, and trehalose is a general response to high osmolality. First, a variety of inorganic or organic solutes can induce this behavior, and, second, these osmolytes are not dependent on the glucose or ammonium ion in the medium. However, when ammonium was replaced by glutamate as the N source, the dominant osmolyte shifted

TABLE 5. Effect of exogenously added NaCl and glycine betaine on the growth of clinical isolates of *P. aeruginosa*[a]

Isolate	Concn of NaCl added to medium (M)	Doubling time (h/generation) with:	
		No addition	Glycine betaine added
JS	0	1.8	1.6
	0.3	2.2	2.1
	0.5	4.2	2.9
	0.7	9.0	3.6
MC	0	2.2	2.0
	0.3	3.3	2.9
	0.5	13	7.1
	0.7	—[b]	13

[a] Cultures were grown in VB medium plus NaCl and 1 mM glycine betaine where indicated.

[b] —, growth under these conditions was not observed reproducibly.

from NAGGN to trehalose. Shifts in osmolyte preference by exogenously added osmoprotectants are well established in the literature. For example, in many bacterial organisms when proline or glycine betaine is added to the growth medium, it is transported and accumulated in the cell, replacing the endogenously synthesized osmolytes (4, 5). What is unusual about *P. aeruginosa* is that glutamate, a nutrient and presumed precursor of NAGGN, causes another endogenously synthesized osmolyte, trehalose, rather than NAGGN, to dominate. It is possible, although unlikely, that glutamate is transported into the cell as a precursor to trehalose. Alternatively, glutamate may behave as a type of environmental signal, controlling the amount of endogenously synthesized osmolytes either at the enzymatic or genetic level. Connections between the osmoregulatory response and a variety of environmental signals such as low temperature, anaerobic conditions (8), or shift to stationary phase (11) have already been reported for *E. coli*. However, to our knowledge, no signal has been found that can regulate the ratio of the endogenously synthesized osmolytes in *E. coli* such as was observed in *P. aeruginosa*.

Just as with *E. coli*, glycine betaine enhances the growth of osmotically stressed *P. aeruginosa* and modulates its intracellular osmolyte composition. However, the contributions of the endogenous osmolytes to the osmotolerance of *P. aeruginosa* must not be minimized. Even at 0.7 M NaCl and with the addition of 1 mM glycine betaine to the medium, the intracellular level of glycine betaine accounts for about two-thirds of the total osmolyte concentration. This result is in contrast with that from *E. coli* in which the addition of glycine betaine obliterates the accumulation of the endogenous osmolytes (14).

Currently emerging is the relationship between the mechanism of osmotic stress adaptation and the process of pathogenicity in *P. aeruginosa*. This organism produces a number of toxins and enzymes that contribute to its pathogenicity (9 [and references therein], 11), and some of these can be linked to osmoregulation. For example, high osmolality itself induces *algD* and *algR*, genes which are required for the production of alginate (1, 7). Also, phosphorylcholine stimulates capsule formation in mucoid *P. aeruginosa* (13). Another striking link is the virulence factor phospholipase C, which hydrolyzes phosphatidylcholine to phosphorylcholine and diacylglycerol. This enzyme is induced by its reaction product phosphorylcholine and by choline, glycine betaine, and dimethylglycine (19). Considering that the lung environment in CF patients is particularly stressful osmotically and also rich in phosphatidylcholine (1, 3, 7, 17, 19), the linkage of osmotic stress adaptation with phospholipase C activity is intriguing. For example, phospholipase C activity of *P. aeruginosa* might also contribute to improved osmotic tolerance of the pathogen in the lung by providing an osmoprotectant. Consistent with this speculation is that osmotically stressed *P. aeruginosa* responds to micromolar concentrations of phosphorylcholine as well as choline and glycine betaine.

Finally, the question arises as to the relevance of this research on *P. aeruginosa* PAO1 to strains found in nature. The results described in this article from two clinical strains isolated from the sputum of the respiratory tracts of CF patients suffering from chronic lung infection indicate that the mechanism of osmotic stress adaptation is nearly identical to that of PAO1. All three strains accumulate high levels of NAGGN, glutamate, and trehalose and are protected against osmotic stress by the addition of glycine betaine to the medium. Hence, research concerned with foiling either NAGGN or glycine betaine accumulation may ultimately yield a useful application in disease management.

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